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Review article

Cell therapy for retinitis pigmentosa: From rats to pigs

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ABSTRACT

Retinitis pigmentosa (RP) is a leading cause of blindness worldwide and lacks effective clinical treatment. Stem cell-based therapy offers a novel experimental therapeutic approach, based on the strategy that transplanted progenitor cells can replace or rescue damaged photoreceptor cells. However, many factors remain to be determined, for example, what is the optimal time to choose for targeting the host tissue during the progression of the degeneration, what the characteristics and potential capacities in different stem cells, do stem cells differentiate into functional daughter cells, and to what degree can host retinal function be restored? We have used Royal College of Surgeons rats and light-induced retinal degeneration minipigs as animal models of retinitis pigmentosa to study the effectiveness of cell transplant therapies and the functional capacity of the host retina. Stem cells from rat retina and bone marrow, neonatal pig, and human fetal retina have been investigated to find the proper donor cells. The dedifferentiation and then redifferentiation of Müller cells following retinal stem cell transplantation may contribute to host visual function and presents a promising line of research.

1. Introduction

Retinitis pigmentosa (RP) is a group of inherited diseases in which progressive photoreceptor dysfunctionis associated with cell loss and eventual retinal atrophy.[1] There are few effective clinical treatments for RP, a disease that affects an estimated 1.5 million individuals worldwide, or other retinal degenerative diseases such as age-related macular degeneration, which is a leading cause of blindness in older individuals.[2] A variety of experimental therapies are under investigation aimed at repairing or rescuing impaired vision, including gene therapy and retinal transplantation. However, gene therapy has proven to be difficult due to problems involved in finding key target genes in the large number of RP-related genes, and the finding that gene therapy can be only used in the early stages of RP.[3] These limitations have recently made the renewed prospect of retinal transplantation or prosthesis more attractive. Various cells, tissues, and devices have been investigated in retinal transplantation, including photoreceptor cells, retinal pigment epithelial (RPE) cells, embryonic or neural stem cells, bone marrow-derived stem cells, retinal stem/progenitor cells, fetal neuroretina (with or without RPE), and retinal prostheses.[4–6] However, there are still many points that require clarification, some of which are as follows. First, what is the best timing to target host degeneration that can produce efficacious functional rescue when using subretinal implantation? Second, what are the characteristics and potential rescuing capacities of different donor cells? Third, can donor cells differentiate into functional daughter cells with appropriate electrophysiological function? Last but not least, how does the graft restore host retinal function after subretinal implantation in RP? We studied these questions in the search for a possible clinical treatment strategy of RP, by using a rat retinal degeneration model, the Royal College of Surgeons (RCS) rats, and light-induced retinal degeneration in minipigs. Our findings are discussed in the following review.

2. Cell therapy for retinitis pigmentosa in animal models

2.1. Surviving ganglion cells—a guide for retinal degeneration stem cell therapy

We investigated the modifications in the density and electrophysiological changes of retinal ganglion cells (RGCs) in RCS rats during retinal degeneration, to see whether there were functional surviving inner layer cells during retinal degeneration. At postnatal week seven (Pn7) and Pn8, the density of the RGC was about 71% of that at Pn3, and by Pn12 this had fallen to ~46%. Thus, although there is a dramatic loss of ganglion cells, a substantial proportion of

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cells are preserved.[7] Whole-cell patch clamp studies showed three RGC action potential discharge patterns in RCS rats: single, transient, and sustained firing. The main discharge pattern was single firing between Pn1 and Pn2, followed later by transient and sustained firing patterns. However, during later stages of retinal degeneration at Pn7–8, 26.7% RGCs lack action potentials in RCS rats, and this proportion had increased to 63.2% by Pn9–12. This suggested that functional RGCs were maintained during the early stages of retinal degeneration, but this was not sustained, even though morphological changes were not apparent.[8] By a new GFP expressing rd10 mutant mouse which is a model of autosomal recessive RP, RGCs showed remarkable preservation of structure, survival, and projections to higher visual centers even after severe damage of photoreceptors.[9] However, all RGCs types showed an undersized dendritic tree, possibly as a consequence of altered visual input.[10] A recent study displayed that in rd10 mouse bipolar cell processes extended into the inner nuclear layer and ganglion cell layer by postnatal month 9.5, which may contribute to the long-term survival of RGCs.[11] Some studies also indicated that in the remodeling of retinal degeneration, unlike second-order neurons such as bipolar and horizontal cells, RGCs appear as a considerably stable population of cells, potentially forming a favorable substrate for restoring vision in RP individuals throughcell therapy.[12–22]

2.2. Fifteen days ion channel maturation for retinal stem cells—benefit for thought in transplantation protocols

The characteristic of the donor stem cells is a key issue when attempting to rescue function in RP retinas. The functionality of the donor cells can be reflected not only by antigen markers, but also cellular activity. Cultured retinal stem cells (rSCs) can differentiate and produce action potentials *in vitro* showing that the maturation of electrophysiological properties after at least 15 days under culture conditions (Fig. 1).[23] These neuron-like cells, presumptive RGCs, developed active electrophysiological features, including inward sodium currents and outward potassium currents. During the process of stem cell differentiation, voltage-dependent ion channels gradually developed in a time-dependent manner and ultimately supported mature action potential firing similar to that

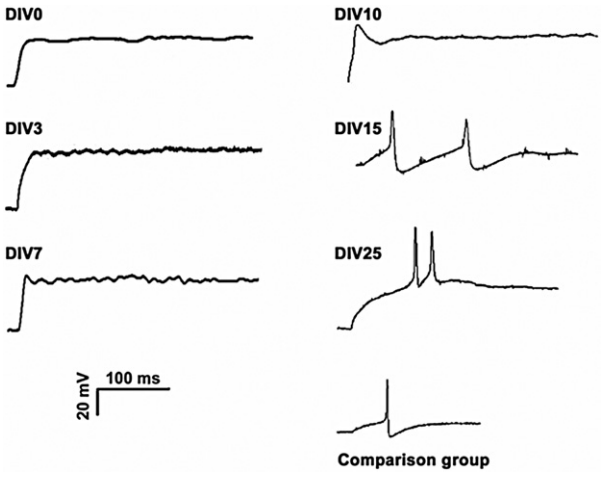

Fig. 1. Differentiated retinal stem cells can generate action potentials. Responses are shown to a 1-second 80-pA intracellular current pulseduring current-clamped recordings after specified day *in vitro* (DIV) culture conditions. Evoked action potentials could not be reliably generated in the cells before DIV10. At DIV10, a broad but shallow single evoked action potential could be recorded, and by DIV25 the action potentials resembled those in the comparison group and had a relatively mature firing pattern. (*Note*. From "Differentiation and production of action potentials by embryonic rat retina stem cells in vitro," by L.F. Chen, Z.Q. Yin, S. Chen, Z.S. Chen, 2008, *Invest Ophthalmol Vis Sci*, *49*, p. 5144–50. With copyrights permission).

observed in the cultured postnatal cells. Importantly, action potentials could be induced but also developed spontaneously. Knowledge of the appearance of voltage-dependent ion channels during development provides a time window for functional stem cell maturation, and this will help to improve the success rate in transplantation protocols if functional recovery is to be achieved.

2.3. Three types of rat stem cells and their subretinal transplantation

We found that three types of stem cells could be incorporated into the degenerating retina and differentiated into rhodopsin positive cells: rat optic cup derived retinal stem cells (OC-rSCs) at embryonic day 12.5, rSCs from embryonic day 17 of Long–Evans rats pretreated by BDNF (rSCs-BDNF), and rat bone marrow stromal cells (rBMSCs) pretreated with fibroblast growth factor (bFGF).

2.3.1. Rat optic cup derived retinal stem cells at embryonic day 12.5

OC-rSCs are easily enriched to 92% by three passages, have a normal diploid karyotype, and exhibit no obvious differences in proliferative rate during eight passages; however, a large number of E12.5 rat embryos are required. To verify that the neurogenic versus gliogenic properties were similar *in vitro* and after transplantation, OC-rSCs were transfected with the Enhanced Green Fluorescent Protein-plasmid (EGFP-plasmid) after passage three and then transplanted into the subretinal space of RCS rats. OC-rSCs were incorporated into the degenerated retina and had differentiated into rhodopsin positive cells.[24] Although the number of double-labeled rhodopsin positive cells was quite low, OC-rSCs offer greater potential for manipulating differentiation into specific retinal phenotypes.

2.3.2. Retinal stem cells from embryonic day 17 Long–Evans rats pretreated by BDNF (rSCs + BDNF)

RCS rats received injections of rSCs, stem cells induced by brain-derived neurotrophic factor (rSCs + BDNF), phosphate-buffered saline (PBS), or BNDF alone.[25] At 1, 2, and 3 months after subretinal transplantation, we found both rSCs eyes and rSCs + BDNF eyes had thicker outer nuclear layers (ONLs) and better photoreceptor maintenance compared to PBS and BDNF injected eyes ($p < 0.01$) at each time point. This suggested that the rate of photoreceptor degeneration and cell death had slowed to some degree. However, outer nuclear layer thickness in rSCs + BDNF transplanted eyes was not significantly different from rSCs transplants alone, suggesting no added benefit from BDNF in terms of cell survival. Although the electroretinogram (ERG) enhancement due to rSCs + BDNF transplants was only apparent in the 1st month after the operation, cellular factors will play an important role in cell replacement therapy in retinal disease by helping to obtain a better survival rate of grafted cells, and by rescuing retinal neurons from further degeneration.

2.3.3. Rat bone marrow stromal cells pretreated with bFGF

Untreated rBMSCs and rBMSCs pretreated with bFGF (composed of phenotypically differentiated retinal neurons) survived for 3 months following transplantation into the subretinal space of RCS and normal control rats.[26] The number of surviving cells in the degenerating RCS rat retina was significantly higher compared to transplants in the normal rat retina. In RCS rats, the bFGF-pretreated cell mixtures containing phonotypically differentiated cells had greater survival rates and ability to migrate compared to the untreated BMSCs. At 1 postoperative month, the latency and amplitude of the Rod-ERG b wave showed significantly more recovery in transplanted rats compared to sham operated rats. A significant increase in the amplitude of the Max-ERG b wave was also observed 1 and 2 months post-transplantation. However, by 3 months the improvement in the Rod-ERG and Max-ERG b wave latency and

amplitude had disappeared.A previous study has shown that a combination of bFGF with BNDF and neuronal growth factor can-induce BMSCs to produce recoverin, PKC, and GFAP.[27] Thus, this bFGF treatment can transiently rescue cells from degeneration of the photoreceptors *in vivo*and partly improve visual function after transplantation for 1 and 2 months.

Therefore, the key elements for stem cells to restore retinal function after subretinal transplantation are high purity, easy access, and pretreatment with cell factors for better survival and differentiation.

2.4. Retinal transplantation in minipigs by neonatal piggy retina or human fetal retina with retinal pigment epithelium

Transplanting only the neuroretina or only the RPE has limited restorative capacity.[6,28] Fetal neuroretina with RPE is potentially more efficacious because it preserves an intact microenvironment or "stem cell niche" for the retinal stem/progenitor cells.[29,30] We developed a transplantation method with an ~67% success ratio in a large animal model, which may be similar to future methods used in clinical practice.[31,32] We examined the ability of intact neonatal minipig and human fetal retina (neuroretina + RPE) to become incorporated into a degenerating retina, its fate after transplantation, and its effect on the host retinal function. Neonatal grafts maintained good viability after laser micro-ablation from the choroid, in contrast to dispase enzyme dissection that appeared to damage the retina. A 12-month follow-up showed that xenograft transplantation had not resulted in any immunological rejection and thus was a safe technique. The human fetal grafts survived and retained characteristics of progenitor precursor cells, such as Chx10 labeling.Multifocal electroretinography (mfERG) of the host retina showed that the P1

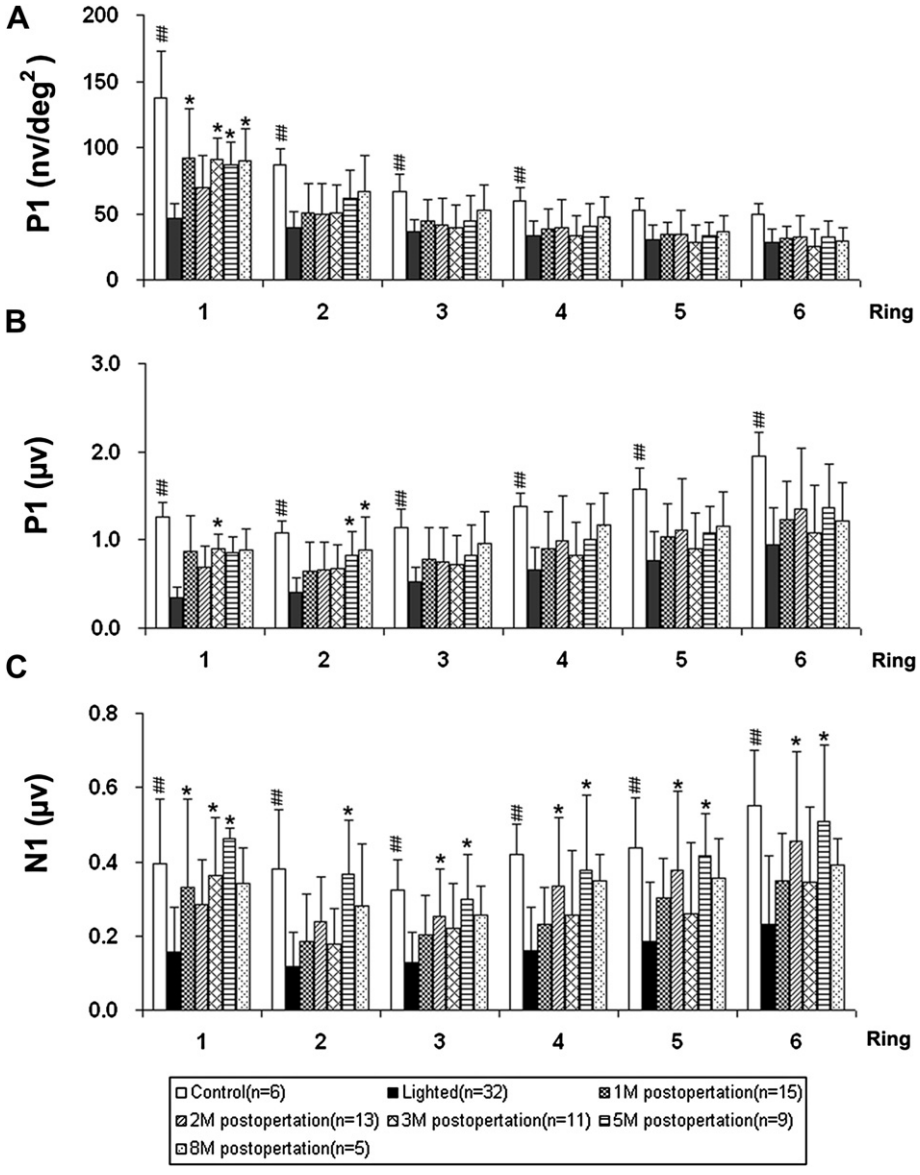

Fig. 2. Multifocal electroretinography (mfERG) analysis of the P1 and N1 wave in normal controls ($n = 6$), at the end of a light-damage period ($n = 32$), and in the light-damaged eyes with a graft after various survival times. The P1 wave *amplitude density* within ring 1 and the *amplitude* in rings 1 and 2 increased, compared with (A, B) their pre-transplantation values. The N1 wave amplitude increased in central (rings 1 and 2) and paracentral retina (rings 3–6) compared with (C) pre-transplantation values. These changes were characterized by two peaks over 8 months: 1st and 3rd to 5th months after the operation. ##$p < 0.05$: control eyes versus eyes at the end of light-damage period. *$p < 0.05$ eyes at the end of light-damage period versus light-damaged eyes with transplants at 1, 2, 3, 5, and 8 months after surgery. (*Note*. From "Rescue from light-induced retinal degeneration by human fetal retinal transplantation in minipigs," by S.Y. Li, Z.Q. Yin, S.J. Chen, L.F. Chen, Y. Liu, 2009, *Curr Eye Res*, *34*, p. 523–35. With copyrights permission).

wave amplitude density within ring 1 and the P1 amplitude in rings 1 and 2 (central retina) increased compared with their pre-transplantation values (Figs. 2A and 2B). The N1 wave amplitude increased in both central (rings 1 and 2) and paracentral retina (rings 3–6) compared with pre-transplantation values (Fig. 2C). The latency of N1 and P1 waves did not show significant changes. These mfERG changes were characterized by two peaks over 8 months: the P1 and N1 wave amplitudes, and P1 wave amplitude density were enhanced in the 1st month after transplantation, maintained in the 2nd month, but subsequently significantly increased in the 3rd and 5th months, and remained until the 8th month. This improvement of host retinal function happened not only in the transplanted area (rings 1 and 2) but also in the areas adjacent to the grafts (rings 3–6) from the 1st to 8th months after transplantation.

2.5. Contribution of Müller cells before and after retinal stem cells transplantation

Müller cells are an integral and important glial component in the normal function of the retina and form a vital part of the regenerative process. Immunocytochemistry in the retina of RCS rats showed that Müller cells express retinal progenitor cell markers in the retinasof chronic degeneration.[33] The number of Chx10 labeled Müller cells dramatically increase in RCS rats that receive rSCs transplants compared with controls (Fig. 3). However, subretinal transplantation of chloromethyl-benzamidodialkylcarbocyanine pre-treated rSCs in RCS rats, showed that only a small number of these grafted cells retain their progenitor cell characteristics.[34] We also found some cells in the inner nuclear layer that could be stained with anti-recoverin rod and cone photoreceptor antibodies and were

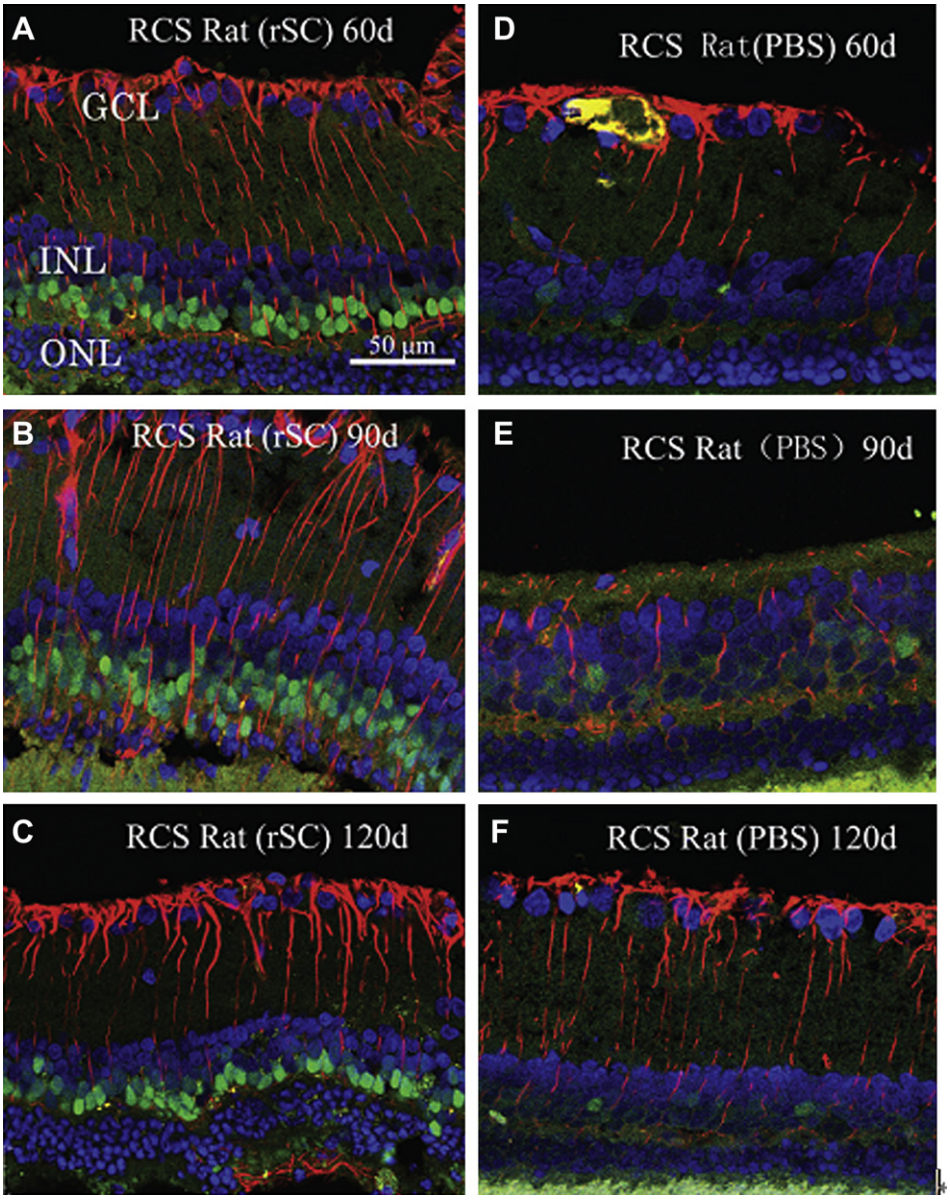

Fig. 3. Increased retinal progenitor cells induced by retinal stem cell (rSC) transplantations. rSC transplants increased Müller cell dedifferentiation. In all micrographs, Chx10 labeling appears green, vimentin labeling appears red, and cell nuclei are blue (DAPI). After rSCs transplantation, the number of dedifferentiated Müller cells (double-labeled red and green) had increased by (A) day 60 (D60), (B) D90, and (C) D120, whereas in the PBS group, (D–F) few cells were double-labeled. DAPI = 4,6-diamidino-2-phenylindole dihydrochloride hydrate. (*Note*. From "Increased Muller cell de-differentiation after grafting of retinal stem cell in the sub-retinal space of Royal College of Surgeons rats," by C. Tian, T. Zhao, Y. Zeng, Z.Q. Yin ZQ, 2011, *Tissue Eng Part A*, *17*, p. 2523–32.With copyrights permission).

double-labeled with vimentin at ~Pn9, Pn13, and Pn17. These findings implied that some of these Müller cells could coexpress a photoreceptor cell marker within and nearby the grafted area. Therefore, Müller cells have the potential to reenter the cell cycle and can differentiate into host photoreceptor cells, thus restoring lost retinal components. Further studies are needed to determine how to maintain the progenitor potential of Müller cells and how rSCs transplants may augment this process of restoring cells, especially photoreceptors, to the damaged retina.

3. Looking into the future

Many studies have shown that the visual function of the host (retinal degeneration models) improved after transplantation of rSCs.[18,35–38] The methods used to determine these results included ERG,multifocal electroretinogram, pupillary light reflex, visual evoked potentials recorded from superior colliculus, and ethological changes due to alteration of visual function.

Our functional investigations show that transplantation of rSCs could delay the progress of retinal degeneration in RCS rats and light-induced retinal degeneration in minipigs. The visual function of the whole retina was improved, not just the regions adjacent to the transplant. Why does this happen? Some studies suggested that graft may form synaptic connections with the host retina on the basis of synaptophysin expression.[18,39] Nevertheless, convincing evidence for a fully functional connection that participates in the transmission of visual signals has not been reported to date. We found that transplantation of rSCs not only increased dedifferentiation of Müller cells in the region of the transplant, but also promoted Müller cells dedifferentiation in nontransplanted region. Transplanted cells can differentiate into photoreceptors and at same time, dedifferentiated Müller cells can differentiate into photoreceptors. That is, after rSC transplantation, not only the direct interaction of graft cells and factors with host retina occurs, but also other factors, such as cytokines released by graft cells, can increase the capacity of host Müller cells to dedifferentiate. Further work needs to done to show that Müller cell dedifferentiation and redifferentiation is a common key event and a viable treatment target in different retinal degeneration diseases. The extent to which rSC transplants contribute to this process remains a major issue in alleviating functional deficits in retinal degenerative diseases.

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